Impacts of activated carbon amendments, added from the start or after five months, on the microbiology and outcomes of crude oil bioremediation in soil

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Abstract

The use of activated carbon (AC) amendments to reduce exposure risks for hydrophobic contaminants like polycyclic aromatic hydrocarbons (PAHs) by adsorption is an innovative soil remediation approach. However, AC amendment side-effects on the pollution biodegradation are poorly understood. This study assessed for optimized nutrient ratio conditions, effects of 5% soil wet weight AC amendments, if added from the beginning or after five months, on the outcomes of one year of crude oil polluted soil bioremediation. CO_2, residual hydrocarbon concentrations and microbial community structure analysis revealed how AC amendment hindered crude oil biodegradation much more, if added from the start, as compared to after 5 months, i.e. after the initial phase of biodegradation. Putative crude oil degrading microorganisms from the genera *Marinobacter, Parvibaculum, Salinibacterium, Muricauda*, and *Alcanivorax* were more sensitive to the AC amendment than those from the genus *Rhodococcus*. *Rhodococci* possess hydrophobic cell walls which may enable them to accumulate hydrocarbons in the AC amended soil, despite of their reduced availability. AC amendment from the start had the highest alkane and total US EPA PAH residues, but was more effective than one year of bioremediation, with or without AC amendment after 5 months, in reducing PAH availability in the soil.

Key words: Activated carbon; Crude oil; Soil pollution; Bioremediation; Next generation sequencing

Declaration of interests: None.

1. Introduction

Bioremediation is frequently utilized to clean-up crude oil polluted soil (Atlas and Philp, 2005). However, high molecular weight PAHs and other complex recalcitrant and toxic compounds in crude oil are hard to biodegrade (Bamforth and Singleton, 2005). Their limited biodegradability leads to their persistence in the environment and possible long-term risks to receptors.

The use of strong adsorbents, such as activated carbon (AC) and biochar, has recently gained attention in the clean-up of soil polluted with PAHs, and other hydrophobic organic compounds (HOCs), due to perceived economic and environmental benefits (Anyika et al., 2015; Bushnaf et al., 2017; Bushnaf et al., 2011; Hale et al., 2012; Kong et al., 2018; Meynet et al., 2012; Meynet et al., 2014; Qin et al., 2013; Ye et al., 2019; Zhang et al., 2016). When added to soil or sediment, AC attracts HOCs, causing these pollutants to transfer to the strong AC sorption sites. Strong sorption limits HOC transfer into the wider environment or uptake by organisms and plants, and thus exposure risks (Denyes et al., 2013; Ehlers and Luthy, 2003). However, while the use of sorbents such as AC limits the pollutant mobility and eco-toxicity, it also reduces microbial bio-accessibility, and consequently may hinder the natural microbial biodegradation processes in soils. This may ultimately result in an increased persistence of HOCs in the environment (Rhodes et al., 2008; Rhodes et al., 2010). A reduced HOC bioavailability could potentially lead to unfavourable shifts in the soil microbial community composition, brought about by the reduced opportunity for growth of HOCs degraders and/or a metabolic switch to other forms of carbon substrates over HOCs. A previous study for an urban, moderately PAH polluted soil found no deleterious effects of AC on indigenous soil HOC degrading bacteria (Meynet et al., 2012), and another study (Ye et al., 2019) reported improved microbial numbers as well as an optimized fungi/bacteria gene copy number ratio following activated biochar addition to a metal and PAH contaminated wetland soil. However, there remains considerable uncertainty about the impacts of AC addition time on soil microbial community composition, and consequently the pollutant biodegradation process, in more heavily polluted soils affected by fresh crude oil spills. It is anticipated that AC effects on the crude oil biodegradation process would be most notable if added immediately after a crude oil spill has occurred, because the pollutant bioavailability, biodegradability and mobility tends to be highest in a freshly polluted soil.

This study aimed to clarify both antagonistic and synergistic interactions of adsorption and biodegradation processes for different approaches to address crude oil pollution in soil, and to identify the best approaches for the use of adsorbents such as AC in this context. A series of batch studies was designed to investigate whether the addition of AC at various times (i.e. from the start or after 5 months) could improve the remediation of crude oil pollution in soils. Geochemical and molecular microbiological techniques (16S rRNA gene sequencing) were integrated to assess the impacts of AC, and AC addition time, on crude oil residuals, the availability of PAHs, and soil microbial communities, including putative petroleum hydrocarbon degraders.

2. Materials and Methods

2.1 Materials

Urban soil was obtained from Exhibition Park, Newcastle upon Tyne, from a surface soil deposit heaped up during a park redevelopment. The total carbon was 3.34 % (organic carbon 2.33%, and inorganic carbon was 1.01%), nitrogen 0.132%, sulphur was 0.05% and soil pH was 7.88. Coconut shell activated carbon (AC) was obtained from Norit (now Cabot Corp, Riga, Latvia) and the properties of this AC have been reported by Han et al. (2015). Briefly, the activated carbon surface area was 975 $m^2\ g^{-1}$, open surface area was 40 $m^2\ g^{-1}$, pore volume was 0.47 $cm^3\ g^{-1}$, micro-pore volume was 0.43 $cm^3\ g^{-1}$, and typical pore size was 37.1 Å.

North Sea crude oil was supplied by British Petroleum plc. The API gravity of the crude oil was 39 degrees and specific gravity (density) was 0.8224. Crude oil analysis (Figure S1 in supporting information) showed that there was a higher concentration of straight alkanes (C_{10}-C_{28}) as compared to the 16 US EPA PAHs. The US EPA PAHs showed a dominating presence of 2, 3 and 4 ring PAHs (93.66% of the total measured PAHs mass, including 45.5% naphthalene, 13.9% fluorene, 29.5% phenanthrene and 4.76% chrysene).

All other chemicals used in the study were of high purity grades, obtained from either VWR International Ltd (Leicestershire, UK) or Sigma-Aldrich (Gillingham, UK).

2.2 Experimental design

Series of batch experiments were set up in 64 mL brown amber vials (Jencons, a VWR Division, Bedfordshire, UK), which were closed with foam plugs, allowing diffusion of oxygen into the vials, or stoppered with Teflon Mininert valves (Supelco, Bellefonte, USA) during headspace measurements. Each treatment was set up in triplicate. A conscious decision was taken not to use sterile controls, as previous experience showed that microbial re-growth is inevitable for long-term experiments, even after double autoclaving. The experimental control batches contained untreated soil (20±2 g wet weight) (‘*Soil’*), or the same amount of soil amended with 1 g of activated carbon (‘*Soil & AC at time zero’*). The experimental batches to simulate crude oil pollution and subsequent remediation contained 20±2 g wet weight of soil spiked with 0.5 mL of crude oil (‘*Soil & oil’*); or spiked with 0.5 mL of crude oil and amended with 1 g of activated carbon added at the beginning (‘*Soil & oil & AC at time zero’*) or after 5 months (‘*Soil & oil & AC at time 5 months’*). Five months were chosen as a reasonable time period to allow the removal of readily biodegradable crude oil components to occur following biostimulation before the addition of AC (Huesemann et al., 2004). Furthermore, since the addition of AC was previously shown to most effectively reduce available concentrations of PAHs in sediments over several months of contact time (Hale et al., 2012; Millward et al., 2005; Zimmerman et al., 2004), all microcosms were incubated for a total period of one year. The mass of crude oil added was equivalent to 20,560 mg/kg of soil to simulate a significant contamination event. A concentrated nutrient solution was prepared by dissolving 13.356 g of NH_4Cl (ammonium chloride) and 1.533 g of KH_2PO_4 in 50 mL distilled water, and 0.5 mL of this solution was dispensed at the start of the experiments into each microcosm vial, including the controls, to simulate biostimulation. Biostimulation provided a C: N: P ratio of 100:10:1 in the batches based on 0.4112 g of crude oil added (with an estimated 84% weight C content).

2.3 Analytical methods

The carbon dioxide was measured each week for 52 weeks by gas chromatography mass spectrometer. Crude oil residue concentrations after 52 weeks of bioremediation were determined by accelerated solvent extraction using hexane:acetone (50:50 v/v), sulphur removal with activated copper, silica gel clean-up and gas chromatography mass spectrometer (Han et al., 2015). The PAH availability and free aqueous concentrations were determined with the help of a passive sampling method (Hale et al., 2010) using polyethylene sheets as passive samplers (VWR International Ltd, Leicestershire, UK). The microbial community was characterized by 16S rRNA gene amplicon sequencing (Bushnaf et al., 2017), which was performed bidirectionally on a Roche 454 GS junior machine (Macropathology, Ltd, Coventry. UK). More detail on the analytical methods used in this study is provided as supporting information.

2.4 Statistical Analysis

Chemical data were analysed using Microsoft Excel version 2010 (Microsoft, Redmond, USA). Student *T-test* was performed using 95% confidence, and two-tailed, independent samples, to compare the effects of the various treatments via the differences in CO_2 production, residual, available and free aqueous pollutant concentrations. Student *T-test* was also used to determine the differences ($p < 0.05$) in relative OTU abundances for bacteria across samples.

The comparison of microbial communities between samples was carried out using PRIMER v6 software (Primer-E Ltd., Plymouth, UK). The Qiime generated OTU table was log transformed and used for the diversity analysis, which includes Bray Curtis dissimilarity metric calculation, based on a pairwise distance (average) and standard deviation (Clarke Robert et al., 2006). The output of the Bray Curtis analysis was used in generating a non-metric multidimensional scaling plot (NMDS), which was integrated with the chemical data. An Analysis of Similarities (ANOSIM) and a Nested ANOSIM (Pearson product moment correlation dissimilarity matrix) was carried out using PRIMER v6 software.

3. Results

3.1 Microbial activity in the microcosms

Microbial activity was inferred by monitoring CO_2 evolution from soil over time (Figure 1). For the soils without crude oil, i.e. controls, there was no significant difference between CO_2 productions from '*Soil & AC at time zero'* and '*Soil'* batches over the entire monitoring period (t-test p value: > 0.05).

With crude oil addition, there was significantly higher CO_2 production from the onset (t-test; $p < 0.05$) for the '*Soil & oil'* and the '*Soil & oil & AC at time 5 months'* batches in comparison to the two controls, *'Soil'* and '*Soil & AC at time zero'*, respectively. The effect of the AC amendment from the start on the crude oil polluted soil respiration is evident from week 5 onwards, with significantly higher (t-test p value: < 0.05) CO_2 production in crude oil batches '*Soil & oil'* and '*Soil & oil & AC at time 5 months'* compared to crude oil batches '*Soil & oil & AC at time zero'* (Figure 1). The CO_2 production from '*Soil & oil & AC at time 5 months'* and '*Soil & oil'* batches was initially similar/comparable, as expected, since treatments were identical up to the adsorbent addition in month 5. Upon the addition of AC there is an indication of slower microbial activity in '*Soil & oil & AC at time 5 months'* compared to '*Soil & oil'* batches (Figure 1) which might be further enhanced over time, but which didn't reach a statistically significant difference during seven months following the AC amendment in this experiment. After 52 weeks, CO_2 production from the '*Soil & oil & AC at time zero*' treatment was approximately 52% and 48% less, respectively, when compared with that of '*Soil & oil*' and '*Soil & oil & AC at time 5 months*' treatments.

3.2 Microbial community composition after 12 months of treatment

After the quality filtering and prior to the concatenating step, 46,241 and 52,189 sequences for the forward and reverse reads respectively were recovered and represented by 797 operational taxonomic units (OTUs, distinguished at species level, i.e. 97% similarity). Figure 2 links the microbial community characteristics to soil respiration over twelve months on an NMDS plot, where the larger bubbles represent higher amounts, and the smaller bubbles represent lower amounts, of carbon dioxide (mg per batch) produced over the 52 week period for the various treatments. The integrated chart shows how higher CO_2 production from the crude oil batches '*Soil & oil*' and '*Soil & oil & AC at time 5 months*', as compared to '*Soil & oil & AC at time zero*' and the controls, are aligned with observed shifts in the microbial community structure. ANOSIM results (based on Bray Curtis similarity coefficients) for the determination of the significance of factors crude oil and AC, confirmed crude oil had a significant effect on the microbial community structure after twelve months with global $R = 0.646$, $p < 0.05$. The results also confirmed that overall, i.e. when including the controls, AC was not a statistically significant determinant in shaping the microbial communities with global $R = 0.145$, $p = 0.085$. However, nested ANOSIM confirmed that AC amendment in crude-oil spiked batches drove changes in the microbial communities (global $R = 0.542$, $p = 0.003$).

The overall relative abundance at phyla (Figure S2 in supporting information), and at genus level (Table 1) showed differences that could be related to crude oil biodegradation, when '*Soil & oil*' and '*Soil & oil & AC at time 5 months*' were compared to the controls ('*Soil*' and '*Soil*

& AC at time zero') and to '*Soil & oil & AC at time zero*'. Relative enrichment was observed in '*Soil & oil*' and '*Soil & oil & AC at time 5 months*' microcosms, as compared to the other treatments and controls, for OTUs from the genera *Parvibaculum, Marinobacter, Salinibacterium, Luteimonas,* and *Alcanivorax.* The genus *Rhodococcus* benefitted in all crude oil spiked microcosms, including '*Soil & oil & AC at time zero*', in comparison to the controls without crude oil (Table 1).

To further illustrate how the different treatments affected soil microbial ecology, the increase or decrease in rank abundance (Table S1 in supporting information) was determined for OTUs distinguished at species level relative to the soil control ('*Original Soil'*, i.e. '*Soil*' which was sampled at the beginning of the batch study). The higher an OTU is ranked in a particular soil treatment, the better it has acclimatised to the treatment conditions, as compared to other species in the soil microbial community. The addition of crude oil only, or crude oil and AC, seemed a favorable condition for *Rhodococcus equi* with an 8 fold increased abundance ranking for both '*Soil & oil*' and '*Soil & oil & AC at time 5 months*' respectively, and a 4 fold increase in '*Soil & oil & AC at time zero*' in comparison to the initial abundance rank in the soil sample. In contrast, no increase in *Rhodococcus equi* relative abundance ranking was recorded after twelve months in the controls without crude oil, '*Soil & AC at time zero*' and '*Soil*'. *Alcanivorax venustensis,* showed a ranked abundance increase of up to 32 fold and 8 fold in '*Soil & oil*' and '*Soil & oil & AC at time 5 months'*, respectively, but only a 2 fold ranked abundance increase was detected for '*Soil & AC at time zero*' and '*Soil & oil & AC at time zero*', in comparison to the initial abundance in the '*Soil*'.

3.3 Crude oil residual concentrations

The extraction of residual crude oil after one year of bioremediation confirmed higher residual concentrations of the sum and individual alkanes, decane to octacosane, in '*Soil & oil & AC at time zero*' in comparison to controls and crude oil batches with AC amendment at time 5 months or no AC amendment (Figure 3 and Table S2 in supporting information). The residual concentration of alkanes was significantly higher (t-test; $p < 0.05$) in *Soil & oil & AC at time zero*' in comparison to the '*Soil & oil & AC at time 5 months*' and '*Soil & oil*' batches. Based on the amount of crude oil alkanes added to the batches, the removal efficiencies of straight-chain alkanes was 29%, 99% and 99% for '*Soil & oil & AC at time zero*', '*Soil & oil & AC at time 5 months*' and '*Soil & oil*' microcosms, respectively.

As examples for the fate of more recalcitrant compounds, the 16 US EPA PAHs residuals (Figure 4 and Table S2 in supporting information) showed a more complex pattern. Overall, there was a significant difference (t-test; $p < 0.05$) and noticeable effect of AC addition time, with microcosms with AC addition at time zero ('*Soil & oil & AC at time zero*') having a 38.5% higher total US EPA PAHs residual concentration in comparison to microcosms with AC addition after 5 months ('*Soil & oil & AC at time 5 months*'). Insofar, the observation for the sum of PAHs mirrored the trend of reduced biodegradation if AC is added from the beginning, as was already observed for the straight-chain alkanes.

However, for the individual PAHs compounds profile (naphthalene - benzo(ghi)perylene) (Figure 4B), there was a remarkable variation in treatment effects with molecular weight (MW). Residuals of high MW PAHs at the end of the twelve months treatments were seemingly lower in the '*Soil & AC at time zero*' control, as compared to the '*Soil*' control microcosms, and there was a corresponding trend of AC amendment effects in the crude oil polluted microcosms, with the '*Soil & oil & AC at time zero*' microcosms seemingly having the lowest high MW PAH residuals.

3.4 Polyethylene concentrations, aqueous concentrations and log K_d estimation

Uptake of PAHs by polyethylene samplers was used as a measure for their availability in the soil after treatment. As is apparent from the PAH availability results (Figure 5), the addition of AC at time zero was able to significantly reduce (t-test; $p = < 0.05$) the PE accumulation of 16

EPA PAHs (average sum and some of the individual high MW PAHs) in treatments '*Soil & oil & AC at time zero*' and '*Soil & AC at time zero*' in comparison to the other treatments. The availability measurements thus confirmed the trends observed for the solvent-extractable high MW PAH residuals (Figure 4B). Correspondingly, the batches '*Soil*' and '*Soil & oil*' without any AC amendment had the highest PE concentration of high MW PAHs. The batches amended with AC after 5 months ('*Soil & oil & AC at time 5 months'*) showed a reduced high MW PAHs availability in comparison to '*Soil & oil'*, but a lesser reduction (t-test, $p < 0.05$), in comparison with the '*Soil & oil & AC at time zero'* treatment.
An estimation of the free aqueous PAHs concentrations at the end of the treatment (Figure S3 in supporting information) was obtained using the PE PAH concentrations and their PE-water partitioning coefficient (K_{PE}) (Hale et al., 2010). The results show a decrease (although not statistically significant) in free aqueous concentrations for the PAHs in treatments '*Soil & oil & AC at time zero*' and '*Soil & AC at time zero'* compared to the others. From the soil and aqueous PAHs concentrations, soil-water partitioning coefficients were also calculated (Figure S4 in supporting information).

4. Discussion

4.1 Microbial activity in the microcosms

Low, and comparable CO_2 productions from the controls '*Soil & AC at time zero'* and '*Soil'* batches over the entire monitoring period (Figure 1) shows that AC per se does not have a deleterious effect on urban soil respiration, in line with previous findings for another urban soil (Meynet et al., 2012). But in crude oil spiked batches, i.e. following addition of a mixture which includes readily biodegradable carbon substrates, the effect of the AC amendment from the start on the crude oil polluted soil respiration is evident from week 5 onwards. There was significantly lower CO_2 production for '*Soil & oil & AC at time zero'*, as compared to '*Soil & oil & AC at time 5 months'* and '*Soil & oil'* batches. This result confirms that AC amendment from the start limits crude oil availability for biodegradation and results in slower mineralisation of crude oil components. Previous studies have already shown how the addition of AC reduces or hinders the rate of hydrocarbon mineralization to CO_2 by limiting the substrate bioaccessibility (Oyelami et al., 2014; Rhodes et al., 2008; Rhodes et al., 2010). In this study, we furthermore demonstrated that AC amendment had a much lesser impact on crude oil polluted soil respiration if added after 5 months ('*Soil & oil & AC at time 5 months'* compared to '*Soil & oil'* batches). This observation of only minor effects confirms our initial expectations that AC amendment would have a lesser impact if added later in the crude oil bioremediation process, i.e. after the removal of the most biodegradable crude oil compounds.

4.2 Microbial community composition after 12 months of treatment

Figure 2 shows how the observed differences in soil respiration for the various treatments were aligned with shifts in the microbial community composition. Relative enrichment was observed in '*Soil & oil*' and '*Soil & oil & AC at time 5 months*' microbial communities, as compared to the other treatments and controls, for OTUs from several genera which include putative hydrocarbon degraders (Table 1 & S1 in supporting information). Members of the genus *Parvibaculum* have been previously detected in PAH polluted soils (Sipilä et al., 2008). The genus *Luteimonas* has been previously linked to the specific degradation or mineralization of benzo(a)pyrene (Jones Maiysha et al., 2014), while members of the genus *Salinibacterium* are known to remove PAHs such as phenanthrene and pyrene (Isaac et al., 2013). The genus *Marinobacter* has been involved in the degradation of a wide range hydrocarbon compounds, which includes crude oil (Shahriari Moghadam et al., 2014) and naphthalene (Hedlund et al., 2001). *Alcanivorax* is a genus which includes voracious alkane degraders (Hara et al., 2003), and *Alcanivorax species* are often predominant at the initial or early stages of crude oil pollution biodegradation (Beesley et al., 2011; Hara et al., 2003; Kasai et al., 2005). The

relative abundance of these genera generally decreases in the order '*Soil & oil'* and '*Soil & oil & AC at time 5 months*' and then '*Soil & oil & AC at time zero'* (Table 1). The findings indicate that addition of AC reduces the growth of these hydrocarbon degraders, and especially so if added from the start. *Alcanivorax venustensis,* an alkane degrader (Fernández-Martínez et al., 2003), showed a ranked abundance increase of up to 32 fold and 8 fold in '*Soil & oil*' and '*Soil & oil & AC at time 5 months',* respectively, but only a 2 fold ranked abundance increase was detected for '*Soil & AC at time zero*' and '*Soil & oil & AC at time zero*', in comparison to the initial abundance in the '*Soil*' (Table S1 in Supporting Information). Members of the genus *Rhodococcus,* on the other hand, had significantly enhanced relative abundance in all of the crude oil batches, including the '*Soil & oil & AC at time zero'* batches, as compared to the controls without crude oil (Table 1). Members of the genus *Rhodococcus* have been reported to degrade crude oil compounds efficiently in the presence of nutrients. Some of the compounds *Rhodococci* degrade that are found in crude oil include a range of saturated hydrocarbons (de Carvalho, 2012; Hamamura et al., 2006) and PAHs such as naphthalene (Boyd et al., 1997). *Rhodococcus equi* has previously been shown to grow on both alkanes (Bouchez-Naïtali et al., 2001) and PAHs (Fijałkowska et al., 1998). *Rhodococci* possess hydrophobic cell walls, which enable them to accumulate hydrocarbons from the environment (De Carvalho et al., 2014; Van Hamme and Ward, 2001). These cell wall properties may be particularly beneficial in AC amended soils, i.e. in the presence of strong sorbents which reduce hydrocarbon availability in the soil. Consequently, *Rhodococci* may be less sensitive to the presence of AC, and gain a competitive advantage in AC amended soils. Overall, the microbiology data confirmed that putative hydrocarbon degrading organisms within the soil microbial community benefitted from crude oil addition, and generally had a more positive response to crude oil addition with no AC, or AC amendment after 5 months, as compared to the AC addition from time zero.

4.3 Crude oil residual concentrations

The extraction of residual crude oil after one year of bioremediation (Figure 3 and Table S2 in supporting information) also confirmed that conventional bioremediation ('*Soil & oil'*) had a much better removal efficiency for straight chain alkanes as compared to AC amendment from the start ('*Soil & oil & AC at time zero'*). But AC addition after five months ('*Soil & oil & AC at time five months'*) had no negative impact on the straight-chain alkane biodegradation. This aligns with the microbial community structure observations (Table 1 and S1 in supporting information), for alkane degrading bacteria like *Alcanivorax venustensis.* Straight-chain alkane biodegradation would likely have occurred early in the crude oil bioremediation process. Hence, AC addition after five months in this study had no detrimental impacts on the ecological competitiveness of alkane degrading soil bacteria and alkane residuals at the end of the bioremediation treatment.

For the 16 US EPA PAHs (Figure 4), the observation for the sum of PAHs mirrored the trend already observed for the straight-chain alkanes, i.e. reduced overall PAH biodegradation, and reduced success of some putative aromatic hydrocarbon degraders like OTUs from the genera *Parvibaculum, Marinobacter, Salinibacterium,* and *Luteimonas,* and *Alcanivorax,* if AC is added from the beginning, with a lesser impact if AC is added after 5 months.

However, individual PAHs compounds profiles reveal a more complex pattern (naphthalene - benzo(ghi)perylene) (Figure 4B and Table S2 in supporting information). Low MW PAHs, such as naphthalene or fluorene, are abundant in crude oil (Figure S1 in supporting information), and more readily biodegradable than high MW PAHs, such as benzo[b]fluoranthene, benzo[a]pyrene, and indeo[1,2,3-cd]pyrene (Bamforth and Singleton, 2005). AC effects on PAH availability for biodegradation are expected to be more notable for the more readily biodegradable, low MW PAHs, as was indeed observed in this study. For high MW PAHs, their relative recalcitrance may mask the effect of a reduced bioavailability in AC

amended soil. Because these compounds are more slowly biodegraded even in the absence of AC, the effect of AC is less notable.

In our study, the high MW PAHs also had a different origin than crude oil. They had very low concentrations in the crude oil itself (Figure S1 in supporting information), but were nonetheless detected at comparable levels in both the crude oil polluted and the control soils without crude oil addition. These results show that they originate predominantly from the soil matrix (Figure 4B). There have been many previous reports of significant background PAHs contamination in sediments and soils in Northeast England (Law et al., 1997; Woodhead et al., 1999). Petrographic analysis has linked these PAHs to the past coal mining and coal combustion activities (Hale et al., 2010). With the general decline of coal related industries in Western Europe, these pyrogenic PAHs compounds are believed to now have been aged in the environment over several decades, becoming a soot-like, hardened substance with lower availability for mass transfer than petrogenic PAHs (Ghosh et al., 2001; Hawthorne et al., 2007). Other reports have also shown seemingly reduced residuals of pyrogenic PAHs and other HOCs following strong sorbent amendment, which is attributed to reduced solvent extractability of PAHs and other HOCs sequestered by the AC (Hale et al., 2012; Hilber et al., 2012; Vasilyeva et al., 2001). Such reduced PAH extractability aligns with a significant exposure risk reduction and is often used as an indicator of AC amendment benefits (Delannoy et al., 2018; Fagervold et al., 2010; Jakob et al., 2012; Ruby et al., 2016). Hence, AC amendment effects on PAH residuals were complex, and outcomes depended on the biodegradability and origin (petrogenic versus pyrogenic) of the different compounds, and the strength of their adsorption by the AC.

4.4 Polyethylene concentrations, aqueous concentrations and log K_d estimation

Over the past decade, the assessment of PAH contaminated soils has moved on from a mere consideration of the total pollutant concentrations towards a more detailed exposure risk assessment, which also considers how soil properties affect the pollutant bioaccessibility, oral bioavailability, and dermal absorption (Xia et al., 2016). In this context, the use of passive sampling has become an important assessment tool (Ghosh et al., 2014). PE samplers are known to mainly accumulate truly dissolved HOCs compounds (Hale et al., 2012). The compounds taken up by PE samplers are believed to be potentially available also for uptake by plants (Jakob et al., 2012), soil-dwelling organisms (Hawthorne et al., 2007) and microbes, including pollution biodegrading bacteria (Meynet et al., 2012). As is apparent from the PAH availability results (Figure 5), the addition of AC from the start was most effective in reducing the PE accumulation of 16 EPA PAHs (average sum and some of the individual high MW PAHs) in both crude oil polluted soils, and also in the original soil, which contained pyrogenic PAHs. AC appeared to have diminished effectiveness as PAH sorbent, if added after 5 months as compared to addition from the start. This observation could hypothetically be attributable to a shorter contact time or the build-up of partially oxidized crude oil metabolites from biodegradation in the first 5 months. Crude oil metabolites could compete for or block the PAHs binding sites of the AC particles (Pignatello et al., 2006), or enhance PAHs desorption and solubility in soil pore-water via the surfactant effect, thus diminishing AC effectiveness. Similar observations were made when translating PAH concentrations in PE samplers into estimated free aqueous PAHs concentrations using PE-water partitioning coefficients. The free aqueous PAH concentration is predominantly made up of low MW compounds giving a different emphasis to the results. The results nonetheless show a decrease (although not statistically significant) in free aqueous concentrations for the PAHs in treatments '*Soil & oil & AC at time zero*' and '*Soil & AC at time zero'* compared to the others. The findings indicate that AC, if added from the beginning, was able to reduce the transfer of the compounds into the aqueous phase, where they would be available for leaching and posing a groundwater pollution risk. For example, naphthalene had the highest soil concentrations for '*Soil & oil & AC at time*

zero', but the lowest free aqueous concentration (although not statistically significantly) in comparison to other crude oil batches. The findings are similar for some of the other low MW compounds, such as fluorene and phenanthrene. Data from field studies (Hale et al., 2012) suggest that PE samplers provide an appropriate assessment of AC impacts on PAH leaching for granular activated carbon amendments, but in powdered activated carbon amended soils, transport by colloidal AC may also be relevant.

From the soil and aqueous PAHs concentrations, soil-water partitioning coefficients could also be calculated (Figure S4 in supporting information) to assess the PAH binding by the soils with and without AC amendment. Compounds with larger K_d are known to bind more strongly to the soil matrix and migrate more slowly and this reduces the tendency of the pollutant reaching receptors in turn lowering potential exposure risks (Mayer et al., 2016). In general, the K_d values for batches amended with AC from the start were larger in comparison to crude oil batches with no AC amendment and AC amendment after 5 months. The AC potential to sequester aromatic hydrocarbons in crude oil contaminated soils appears to be significantly reduced after a period of crude oil biodegradation (i.e. 5 months in this study), presumably due to the interference of metabolites from the crude oil biodegradation with PAH sorption by AC, as well as shorter overall contact time. Hence, larger AC amendment doses, or a more extensive preceding period of biodegradation, beyond the 5 months investigated in this study, may be required to first minimize interfering metabolite residuals before successfully mopping up persistent residues with AC at the end of crude oil polluted soil bioremediation.

5. Conclusions

AC amendment slowed down the bioremediation of crude oil, and relative abundance gains of hydrocarbon degrading bacteria, like *Parvibaculum, Marinobacter, Salinibacterium, Luteimonas,* and *Alcanivorax,* and much more so if AC was added at the start, as compared to after 5 months of biostimulation. Crude oil degrading bacteria from the genus *Rhodococcus* were less sensitive to the AC amendments. If remediation outcomes are assessed based on total crude oil residuals, AC amendment from the start is likely to result in a disappointing outcome. An exception could be highly weathered soil pollution, as is exemplified for the background pyrogenic PAH pollution in the urban soil investigated in this study. If remediation goals are formulated in terms of reducing the pollutant toxicity, mobility and availability for uptake by receptors, then AC amendment from the start can be an effective remediation strategy, even if it results in higher residual pollutant concentrations in the soil. In this study AC amendment after 5 months was less effective than AC amendment from the beginning in reducing PAHs free aqueous concentrations and availability. More complete bioremediation or higher AC doses may be needed to effectively mop up residuals, and maximize mutual benefits of the two remediation strategies.

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Table 1. Potential hydrocarbon degraders detected from amplicon sequence libraries, and distinguished at genus level.

Taxon level: Genus	Average OTU relative abundances					
	Soil (control 1)	SoilAC (control 2)	SoiloilAC time zero	SoiloilAC 5 months	Soiloil	Previous reported HC degradation activities
Luteimonas	$3.14\ 10^{-3}$	$3.66\ 10^{-3}$	$8.26\ 10^{-3}$	$1.15\ 10^{-2}$	$1.97\ 10^{-2}$	Benzo (a) pyrene (Jones et al., 2014), Luteimonas Mephitis detected in PAH mixtures (Bacosa and Inoue, 2015).
Marinobacter	$3.99\ 10^{-3}$	$7.66\ 10^{-3}$	$8.12\ 10^{-3}$	$2.44\ 10^{-2}$	$1.87\ 10^{-2}$	Crude oil (Shahriari Moghadam et al., 2014), Naphthalene (Geiselbrecht et al., 2001), Fluoranthene, chrysene, benzo (a) anthracene (Vila et al., 2010) Aliphatic HC hexane, pristine, eicosane (Brito et al., 2006). Hexadecane (Fernández-Martínez et al., 2003), Utilization of tetradecane (Fernández-Martínez et al., 2003)
Parvibaculum	$9.50\ 10^{-5}$	$1.82\ 10^{-3}$	$7.47\ 10^{-3}$	$1.19\ 10^{-2}$	$1.95\ 10^{-2}$	Alkanes (Austin et al., 2013) and PAHs (Lai et al., 2011; Sipilä et al., 2008)
Rhodococcus	$1.31\ 10^{-3}$	$3.01\ 10^{-3}$	$1.04\ 10^{-2}$	$2.42\ 10^{-2}$	$1.62\ 10^{-2}$	Efficient removal of naphthalene (Boyd et al., 1997). Hexadecane (Hamamura et al., 2006) Fluoranthene and pyrene (Dean-Ross et al., 2002)
Salinibacterium	$7.34\ 10^{-4}$	$3.05\ 10^{-4}$	$7.52\ 10^{-4}$	$5.77\ 10^{-3}$	$1.62\ 10^{-2}$	Utilize phenanthrene and pyrene under aerobic conditions (Isaac et al., 2013)
Muricauda	NA	$1.17\ 10^{-3}$	NA	$1.56\ 10^{-2}$	$2.43\ 10^{-2}$	Naphthalene, phenanthrene, dibenzothiophenes and carbazoles (Jiménez et al., 2011)
Alcanivorax	$7.75\ 10^{-5}$	$1.60\ 10^{-4}$	$1.12\ 10^{-4}$	$2.79\ 10^{-3}$	$2.12\ 10^{-2}$	Aliphatic hydrocarbons (branched and unbranched alkanes) (Hara et al., 2003) *Alcanivorax venustensis* utilized- Hexadecane and tetradecane (Fernández-Martínez et al., 2003)

Figure 1: CO_2 emanating from soil microcosms. AC indicates activated carbon amendment. Controls: Soil, Soil & AC at time zero. Crude oil spiked vials: Soil&oil&AC at time zero, Soil&oil&AC at time 5 months, Soil&oil. Error bars represent ± 1 standard deviation (SD=3) for the mean of three replicates.

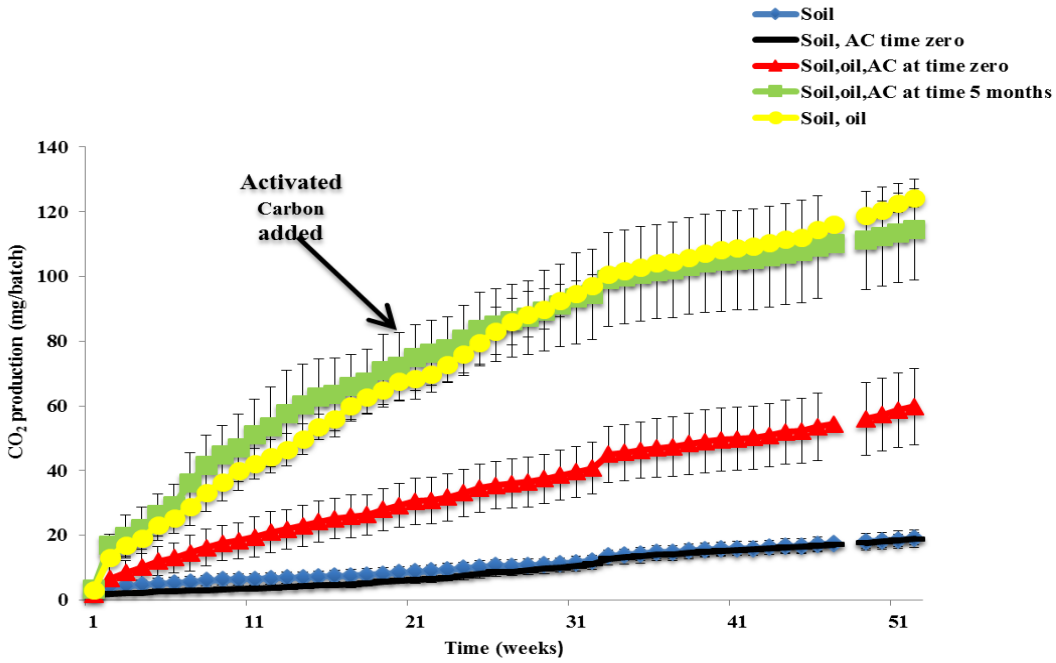


Figure 2: Integrated multi-dimensional scaling plot of 16S rRNA gene amplicon sequencing data and CO_2 data. The blue bubble size indicates the amount of CO_2 produced by month 12 (mg/batch).

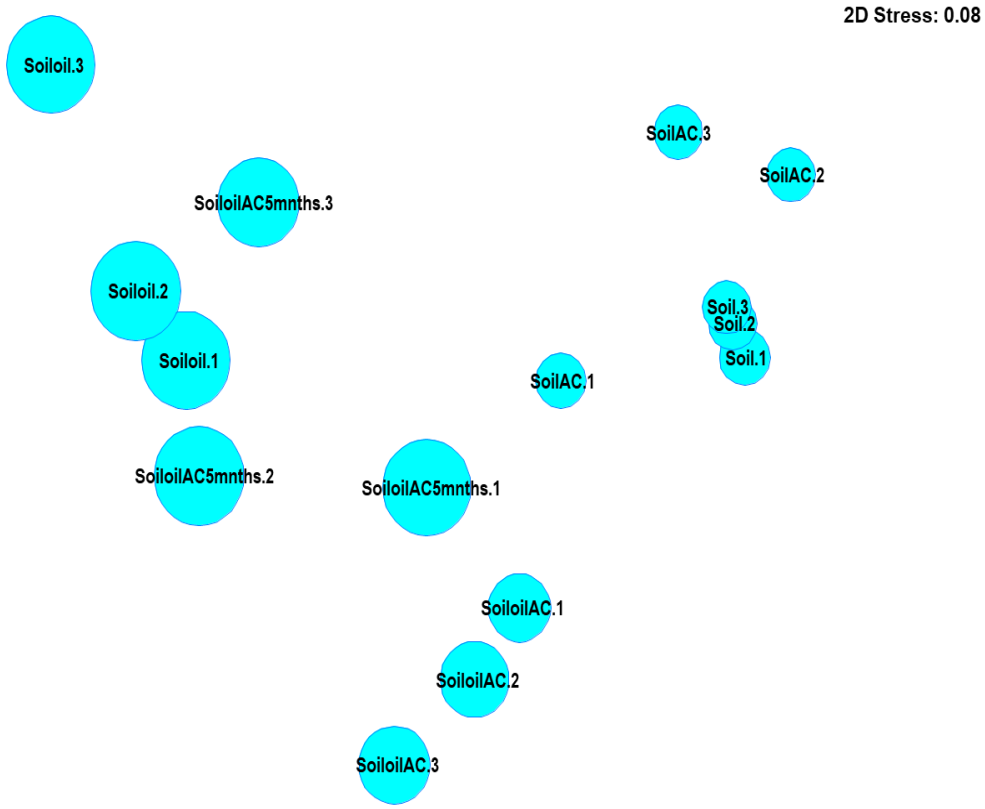
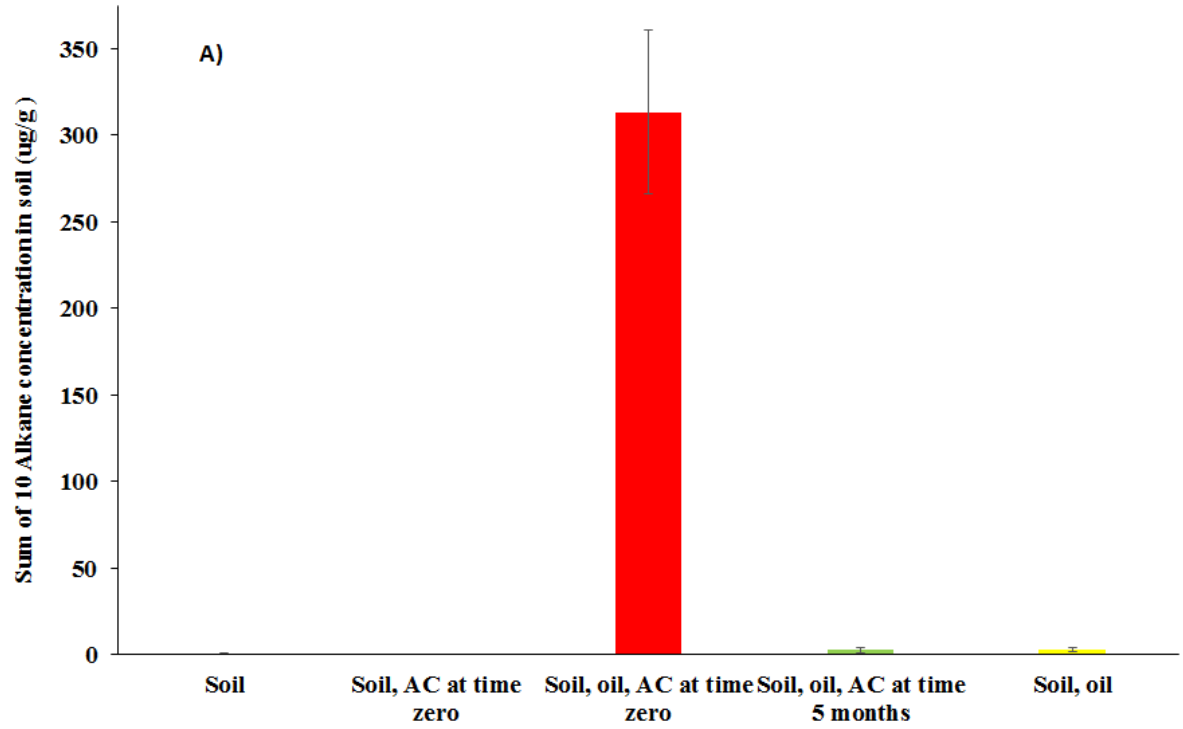


Figure 3. Soil concentration of alkanes after 52 weeks of biodegradation with and without AC amendments. Average alkane concentrations detected in batches. A: sum of the alkane concentrations per treatment, B: concentration of individual alkane compounds per treatment. Error bars represent ±1 standard deviation (SD=3) from the mean of three replicates.



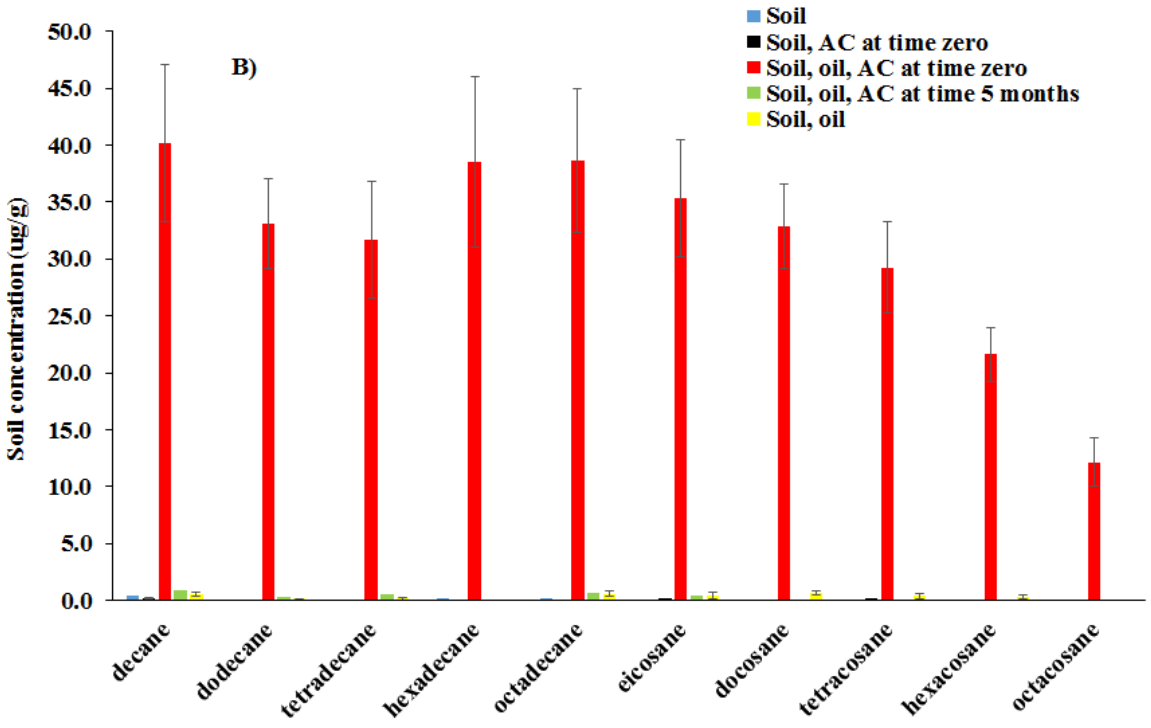
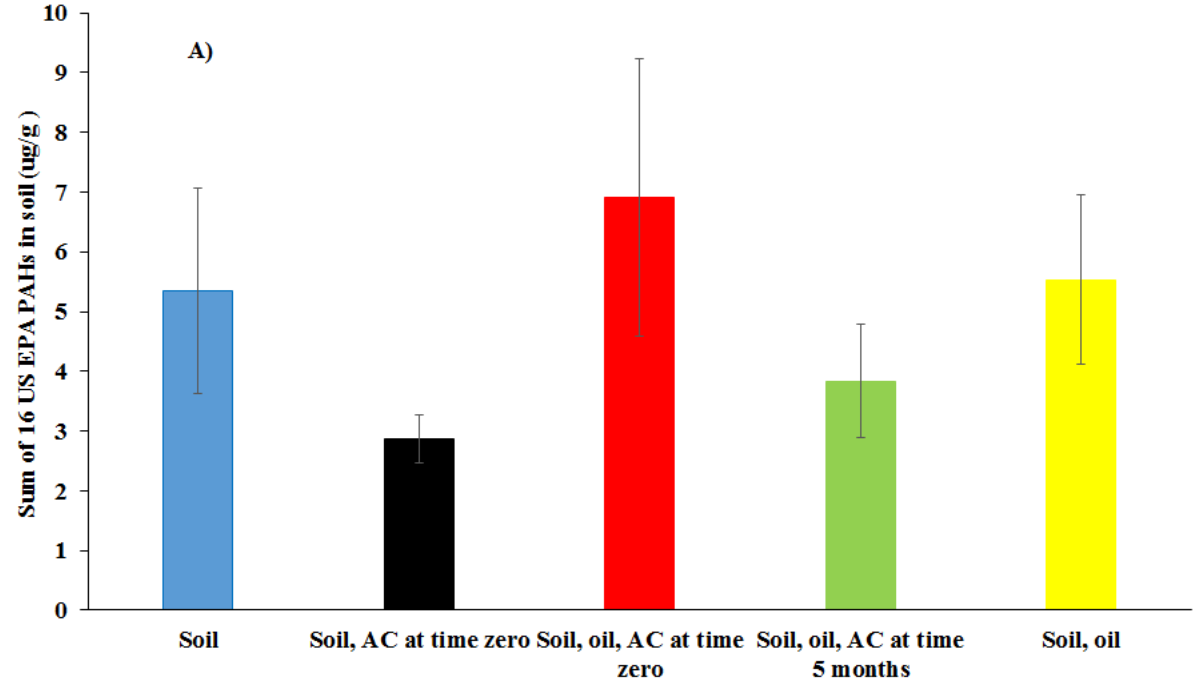


Figure 4: Soil concentration of 16 US EPA PAHs after 52 weeks of biodegradation with and without AC amendments. Average PAHs concentrations detected in batches. A: sum of the PAHs concentrations per treatment, B: concentration of individual PAHs compounds per treatment. Error bars represent ±1 standard deviation (SD=3) from the mean of three replicates.



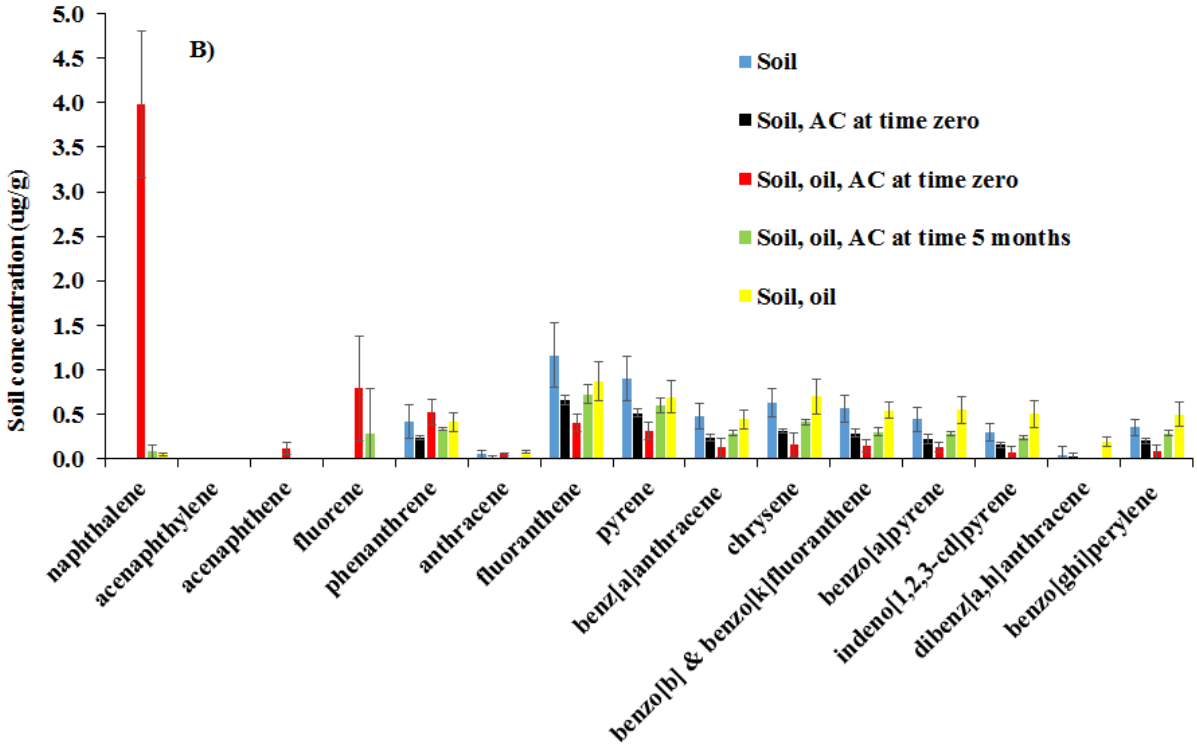
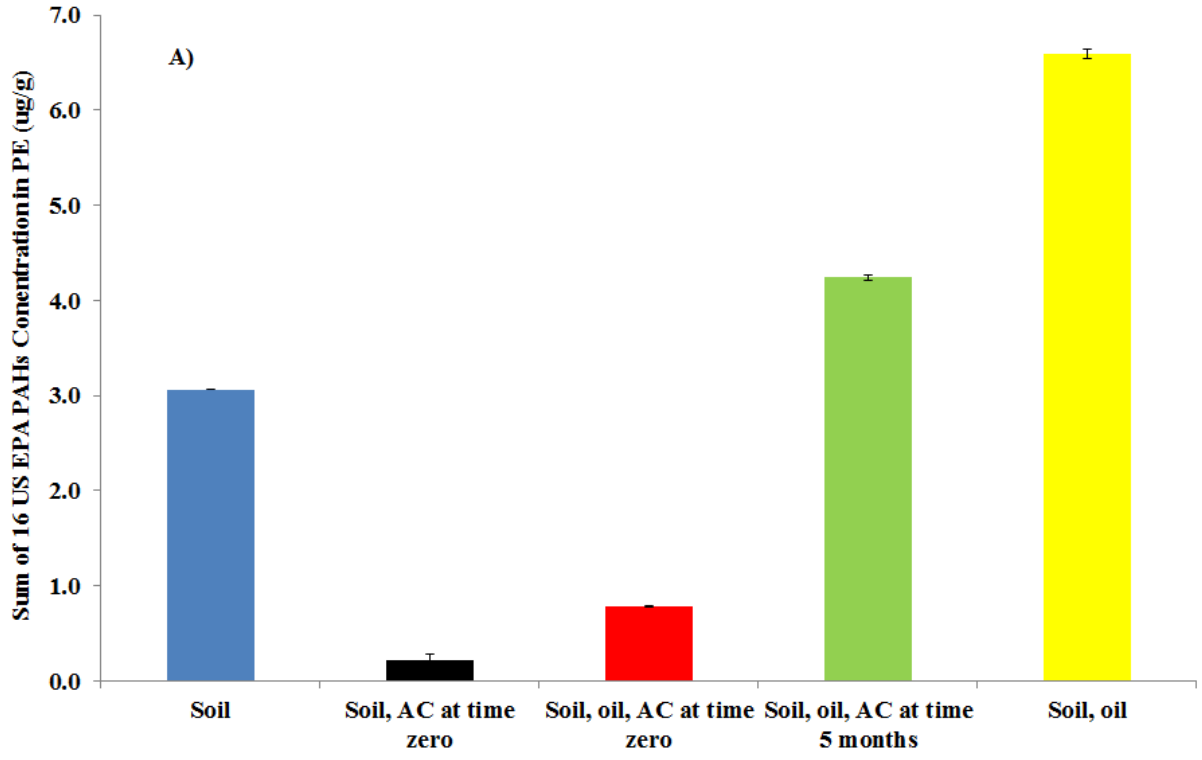
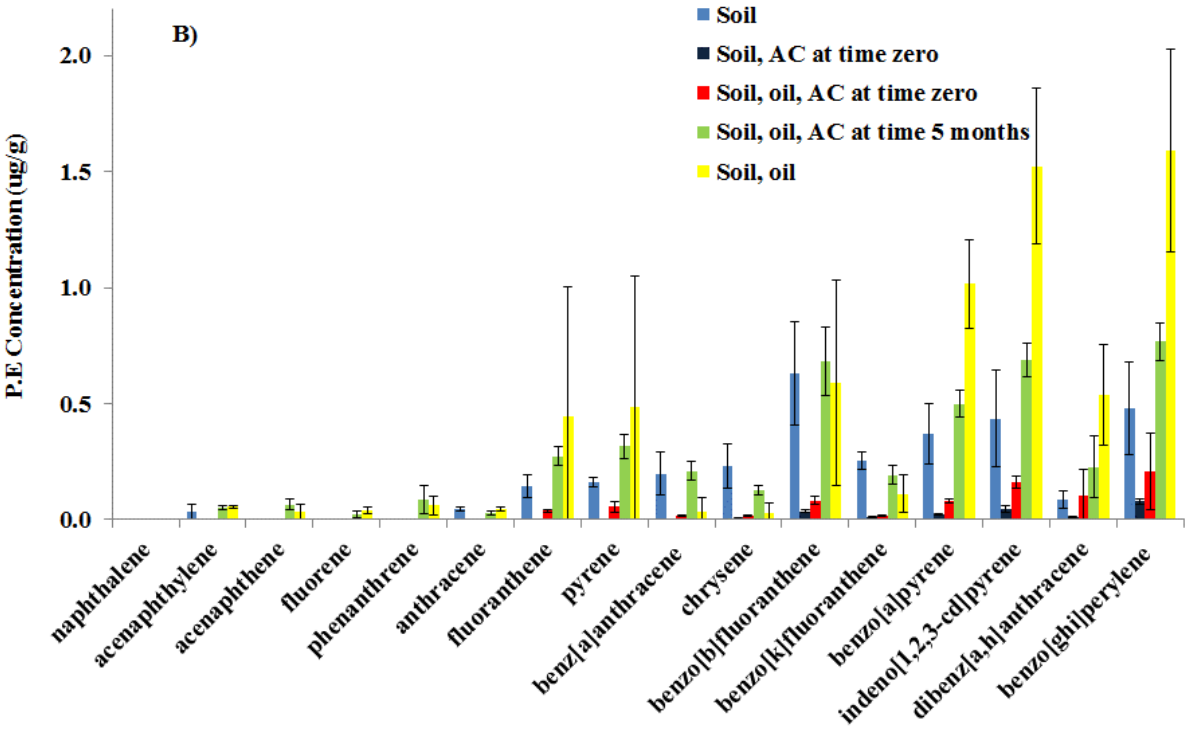


Figure 5: Polyethylene sampler's uptake of PAHs after 52 weeks of biodegradation or AC amendments. Average PAHs polyethylene concentrations detected in batches. A: sum of PAHs compound concentrations per treatment, B: concentration of individual PAHs compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates.





Supporting Information

Impacts of activated carbon amendments, added from the start or after five months, on the microbiology and outcomes of crude oil bioremediation in soil

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Materials and Methods

Soil gas sampling and analysis

The carbon dioxide was measured each week for 52 weeks. Airtight Teflon Miniert valves replaced the foam plugs for 2 days before the measurement to allow the build-up of carbon dioxide in the vial headspace. Carbon dioxide CO_2 production was analysed by injecting 100 µL of headspace gas into a 8060 gas chromatography attached to a Fisons MD800 mass spectrometer. The settings are as follows: 70 eV, filament current 3.6 A, emission current 150 µA, current source 600 µA, temperature 150 ^{0}C, multiplier voltage 300 V, at 3 turns. Gases were separated with a HP-PLOT-Q capillary column (30 m x 0.32 mm i.d) packed with 20 µm Q phase (Agilent Technologies, Palo Alto, USA) and the GC was sustained isothermally at 35 ^{0}C, with helium carrier gas (30 mL/min flow rate, 65 kPa pressure, split at 100 mL/min).

Soil (Solid phase) concentrations determination

5 g of soil was mixed thoroughly with furnaced sea sand and transferred into accelerated solvent extractor (ASE) cells. Cellulose filter paper was placed at the top and bottom of the cells and topped up with more sea sand at both ends prior to sealing with preassembled extractor caps. Extraction was carried out on a Dionex ASE machine (ThermoFisher Scientific, Waltham, USA) with hexane:acetone (50:50). The extraction program was: 5 minute heating up time, 5 minute static period, 1500 psi cell pressure, 100 ^{0}C oven temperature, 100% flush volume, 120 seconds purge time, 3 cycles and 40 mL of extraction solvent. Extracts were collected in 64 mL glass vials sealed with Teflon septum (sterile) caps. Activated copper was used for sulphur removal. Extracts were blown-down using nitrogen in a Turboevaporator (Caliper, Hopkinton, United States), and solvent-switched to cyclohexane. Clean up and fractioning were carried out using column chromatography with 3 g of silica gel topped by a spatula full of sodium sulphate, and 15 mL of hexane was used to elute the aliphatic fraction, followed by 15 mL hexane:dichloromethane (60:40 v/v) to elute the aromatic fraction. Quantification of hydrocarbons by GC-MS was done as previously described (Hale et al., 2010b; Han et al., 2015), using squalene and deuterated phenanthrene as surrogate standards. The calibration standards were obtained from Sigma-Aldrich (Gillingham, UK) and contained 10 alkanes and 16 US EPA PAHs to quantify selected components of the aliphatic and aromatic hydrocarbon fractions, respectively. Four concentration points were prepared with the standards to build the calibration curves. All solvents used in the study were of high purity grades, obtained from either VWR International Ltd (Leicestershire, UK) or Sigma-Aldrich (Gillingham, UK), and solvent blanks were run prior to each analysis to check for contaminants.

Free aqueous concentrations determination

After 52 weeks, the remaining soil from each treatment was homogenized. One portion was preserved in sterile phosphate-buffered saline and kept at -20 °C for microbial analysis. The remaining portion was used to investigate the availability of hydrocarbons. Polyethylene (PE) sheets (VWR International Ltd, Leicestershire, UK) were cut into thin strips (300±0.1mg) and cleaned by soaking in hexane:acetone (50:50 v:v) for 24 hours in aluminium covered glass beakers in the fume cupboard. The remaining soil was weighed and combined with 40 ml of distilled water and one sampler (cleaned PE strip) was added to each vial. Biodegradation was inhibited via the addition of sodium azide (0.4 mL, 1%, Sigma Aldrich). The vials were placed horizontally and equilibrated for 4 weeks by continuous agitation on an orbital shaker at 130 rpm. PE extraction was carried out according to Han et al. (Han et al., 2015). The clean-up and analysis of extracts followed the procedure outlined above. The aqueous pollutant concentrations (W_C) was obtained using the PE concentrations (PEc) and the PE to water partitioning coefficient (K_{pe}) (Hale et al., 2010a), according to

$$Wc = PEc / K_{pe}$$

The residual soil concentrations (Cs) and estimated water concentrations (Wc) were used in estimating the solid-water distribution coefficient (K_d) as described by Zimmerman et al. (Zimmerman et al., 2004) according to

$$K_d = \frac{C_s}{Wc}$$

Microbial Assay

The microbial community was characterized by 16S rRNA gene amplicon sequencing. From the PBS-preserved samples, 2 mL of soil was used to extract genomic DNA using a Fast-DNA Spin kit (MP Biomedicals, UK) following the manufacturer's instructions. PBS was removed by centrifugation (13,000 rpm, 2 mins) prior to the extraction. Each sample was differentiated by adding unique 8 base pairs barcodes to both forward and reverse primers (5'- end for forward and reverse via a GA linker). GS FLX titanium adapter A (5' - CGTATCGCCTCCCTCGCGCCATCAG – 3') and adapter B (5' – CTATGCGCCTTGCCAGCCCGCTCAG – 3') were also attached to the primers. The PCR reaction mix was composed of fast start high fidelity (HF) buffer (containing $MgCl_2$), fast start HF enzyme blend (Roche diagnostic, Germany), PCR grade nucleotide mix (dNTPs, Roche) and nuclease water. PCR program settings were: initial denaturation at 95°C for 4 min, followed by 25 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 45 s and extension at 72°C for 1 min; and a final extension cycle at 72°C for 7 min. The amplicon libraries were then purified using a QIAquick purification kit (QIAGEN, Crawley, UK), quantified on a Qubit 1.0 fluorometer (Invitrogen, USA), using the Qubit dsDNA HS assay kit (Invitrogen, USA), and pooled equimolarly. Sequencing was performed bidirectionally on a Roche 454 GS junior machine (Macropathology, Ltd, Coventry. UK). The sequencing was performed bi-directionally (from 515f and other from 926r).

Sequencing data were analysed using Qiime version 1.8. Briefly, the sequencing raw reads were initially demultiplexed and quality filtered using a minimum sequencing length of 200 nucleotides and a minimum quality score of 20. No ambiguous bases (N) exceeding the limit of 6 were allowed, exact matches to barcodes in mapping files and sequences containing homopolymers longer than 6 were discarded. The remaining sequences were then denoised (Reeder and Knight, 2010) using a cluster of 15 CPUs to correct for sequencing errors and to prevent a high amount of erroneous Operational Taxonomic Units (OTU). The denoised sequences were inflated, forward and reverse reads were merged prior to reintegration into the QIIME pipeline. Clustering of sequences into OTUs was done at 97% similarity using the Uclust algorithm. OTU taxonomy assignment was carried out at a 80% threshold with the RDP classifier (Wang et al., 2007) and aligned using PyNAST (Caporaso et al., 2010). Chimera sequences were removed using the QIIME's Chimera Slayer (Haas et al., 2011). The sequences were filtered using a Lanemask file. The representative aligned filtered sequences were used in generating a Newick phylogenetic tree using FastTree2 (Price et al., 2010) for further analysis. OTU abundance tables for each sample at different taxonomic levels were generated using selected representative sequences and their taxonomic assignments.

Table S1: Operational taxonomic unit (OTU) increases or decreases in their ranked relative abundance of different treatments compared to the original soil (O.Soil). The OTUs were differentiated at species level (clustering at 97% similarity), but OTU taxonomy assignment was not always possible at species level. The highest level classification of each OTU is indicated in the first column. Only OTUs with significant rank abundance changes were included in the table.

OTU (Highest level classification)	Relative abundance rank increase for different treatments compared to the original soil (O.Soil).+ 2-fold, ++ 4-fold, +++ 8-fold, ++++ 16-fold, +++++ 32-fold, ++++++ 64-fold or greater (and vice versa for reduction, minus -)					
	O.Soil (rank)	Soil	Soil & AC	Soil & oil & AC time zero	Soil & oil & AC 5 months	Soil & oil
Rhodococcus equi	99	-	-	++	+++	+++
Flavobacteriaceae	174	++	++++	++++	+++++	++
Flavobacterium	337	+	++	++	++++	+++
Alphaproteobacteria	105	+	+++	+++++	+++++++	++++++
Skermanella	476	-	-	-	+	+++
Altererythrobacter epoxidivorans	138	+	+	+	++	+++
Marinobacter bryozoorum	107	+	++	++	++++	++++
Alcanivorax venustensis	476	-	+	+	+++	+++++
Acinetobacter	69	-	++	++++++	+++++	++++++
Luteimonas	175	-	-	++	++	+++

Table S2: Percent mass of straight-chain alkanes and US EPA PAHs which was degraded or irreversibly sorbed over 12 months relative to the initial masses in the batches. Uncertainties are indicated as ± 1 standard deviation, ≈ 0% means that the % reduction was not significantly different from zero. Concentrations of acenaphthylene, benzo[k]fluoranthene and dibenz[a,h]anthracene were too low to establish the mass balance.

Compound	Soil & oil	Soil & oil & AC after 5 months	Soil & oil & AC at time zero
Decane	99.4±0.3%	98.9±0.3%	49.1±8.7%
Dodecane	99.9±0.1%	99.5±0.3%	53.6±5.5%
Tetradecane	99.8±0.2%	99.1±0.7%	49.9±8.1%
Hexadecane	100.0±0.0%	100.0±0.0%	31.5±13.3%
Octadecane	98.8±0.5%	98.6±1.2%	15.4±13.7%
Eicosane	98.9±0.8%	99.0±0.4%	≈ 0%
Docosane	98.1±0.6%	100.0±0.0%	≈ 0%
Tetracosane	98.6±0.8%	100.0±0.0%	≈ 0%
Hexacosane	98.3±1.0%	100.0±0.0%	≈ 0%
Octacosane	100.0±0.0%	100.0±0.0%	≈ 0%
Naphthalene	99.9±0.0%	99.8±0.2%	89.6±2.1%
Acenapthene	100.0±0.0%	100.0±0.0%	89.3±6.0%
Fluorene	100.0±0.0%	97.5±4.3%	93.3±5.0%
Phenanthrene	98.3±0.4%	98.7±0.1%	97.9±0.6%
Anthracene	87.3±2.7%	100.0±0.0%	91.0±2.6%
Fluoranthene	59.6±10.4%	66.3±4.8%	81.0±4.6%
Pyrene	69.8±7.9%	74.1±3.6%	86.3±4.5%
Benzo[a]anthracene	46.3±12.8%	65.0±2.9%	84.9±13.1%
Chrysene	84.6±4.2%	90.9±0.6%	96.6±3.0%
Benzo[b]fluoranthene	≈ 0%	46.9±8.3%	73.6±11.7%
Benzo[a]pyrene	≈ 0%	36.2±4.9%	70.8±10.9%
Indeno[1,2,3cd]pyrene	≈ 0%	20.4±7.6%	75.2±22.1%
Benzo[ghi]perylene	≈ 0%	19.3±8.3%	76.8±20.8%

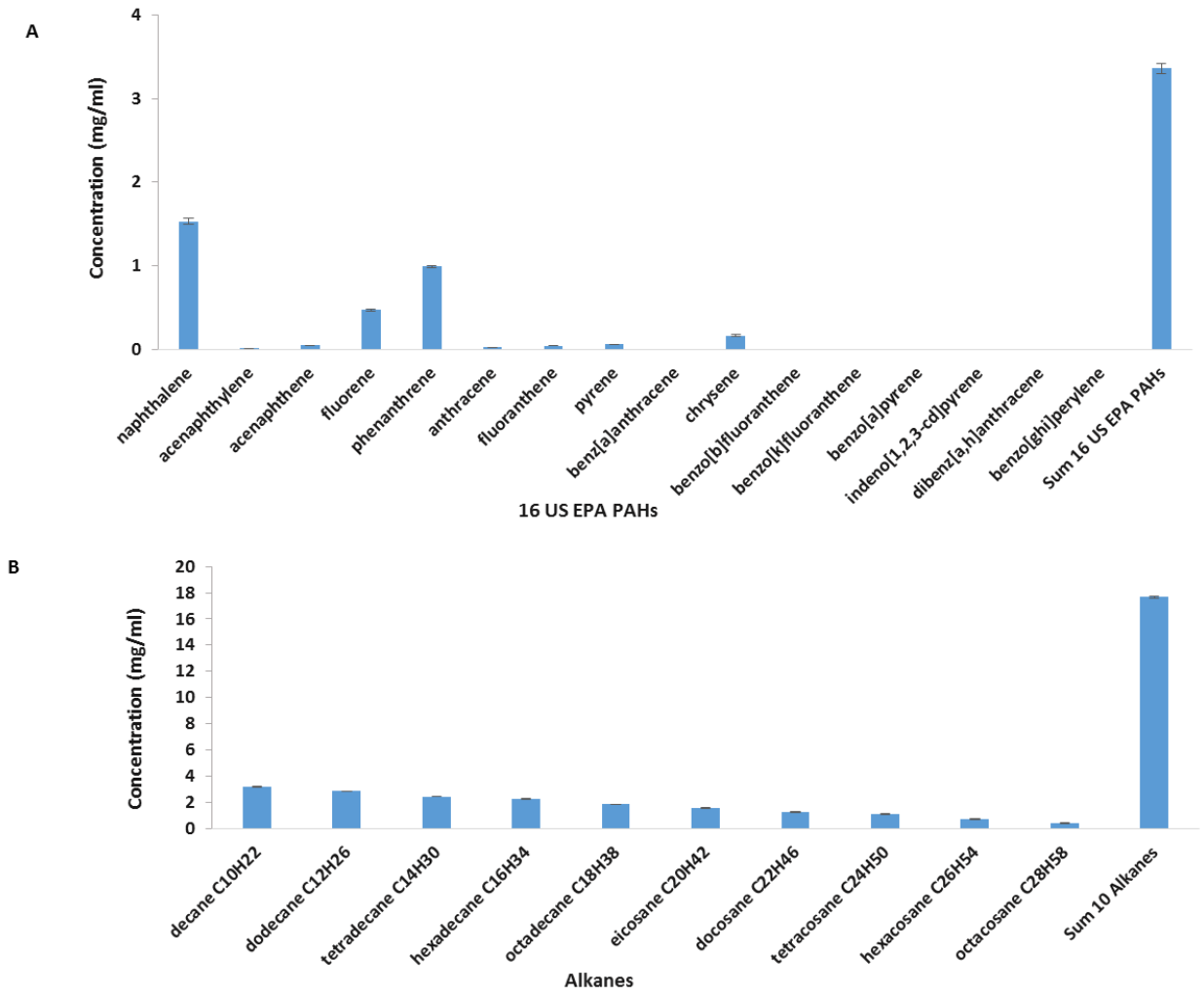

Figure S1. Analysis of crude oil (North Sea) used in batch experiment. A: 16 US EPA PAHs, B: 10 alkanes. Compounds with no visible bars were below detection limit. Error bars ± 1 standard deviation (SD, n=3)

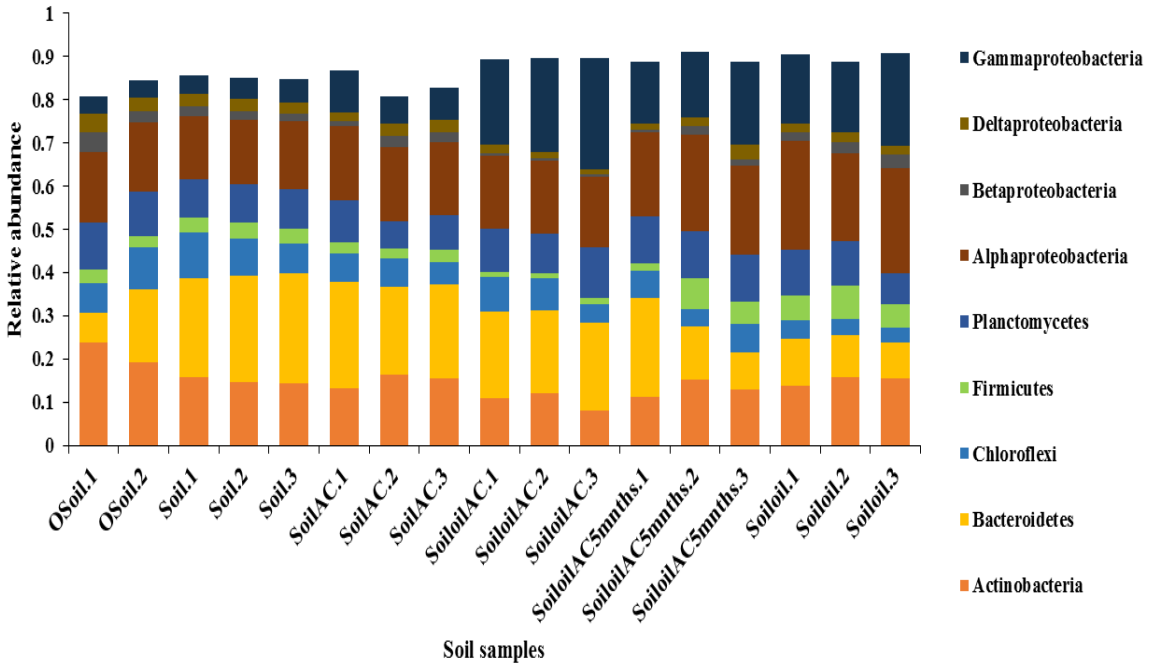

Figure S2. Relative abundance of phylogenetic groups in treatment soils, taxonomy assigned at 80% bootstrap cut-off using the RDP classifier. Controls, AC amended and AC un-amended crude oil soils. Labelled OTUs (distinguished at phyla level) account for ≥ 3.0% of all classified sequences across all samples.

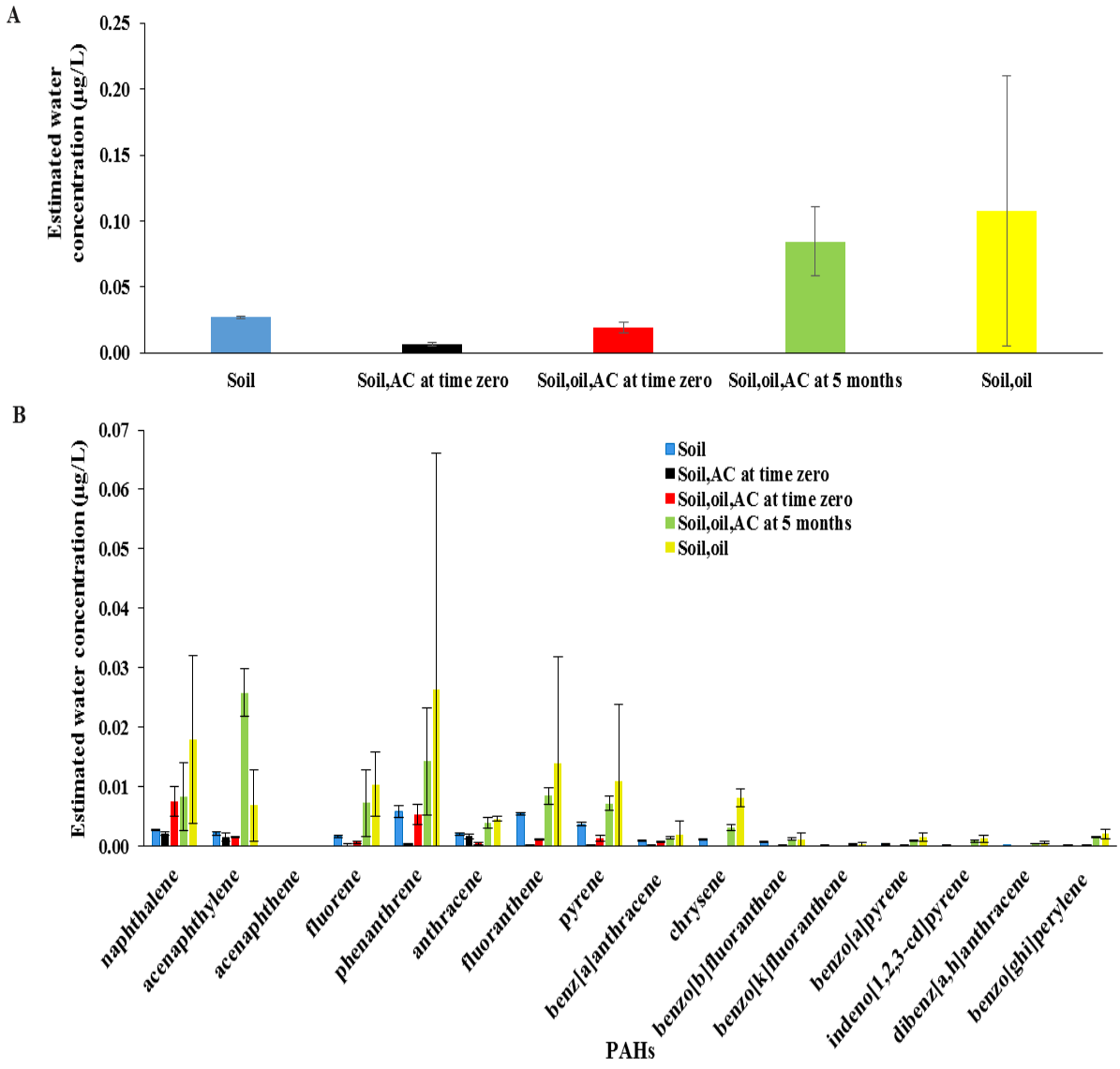

Figure S3. Free aqueous concentration of PAHs after 52 weeks of biodegradation or AC amendments. A: sum of the PAHs concentrations per treatment, B: concentration of individual PAHs compounds per treatment. Error bars: represent ±1 standard deviation (SD=3) from the mean of three replicates

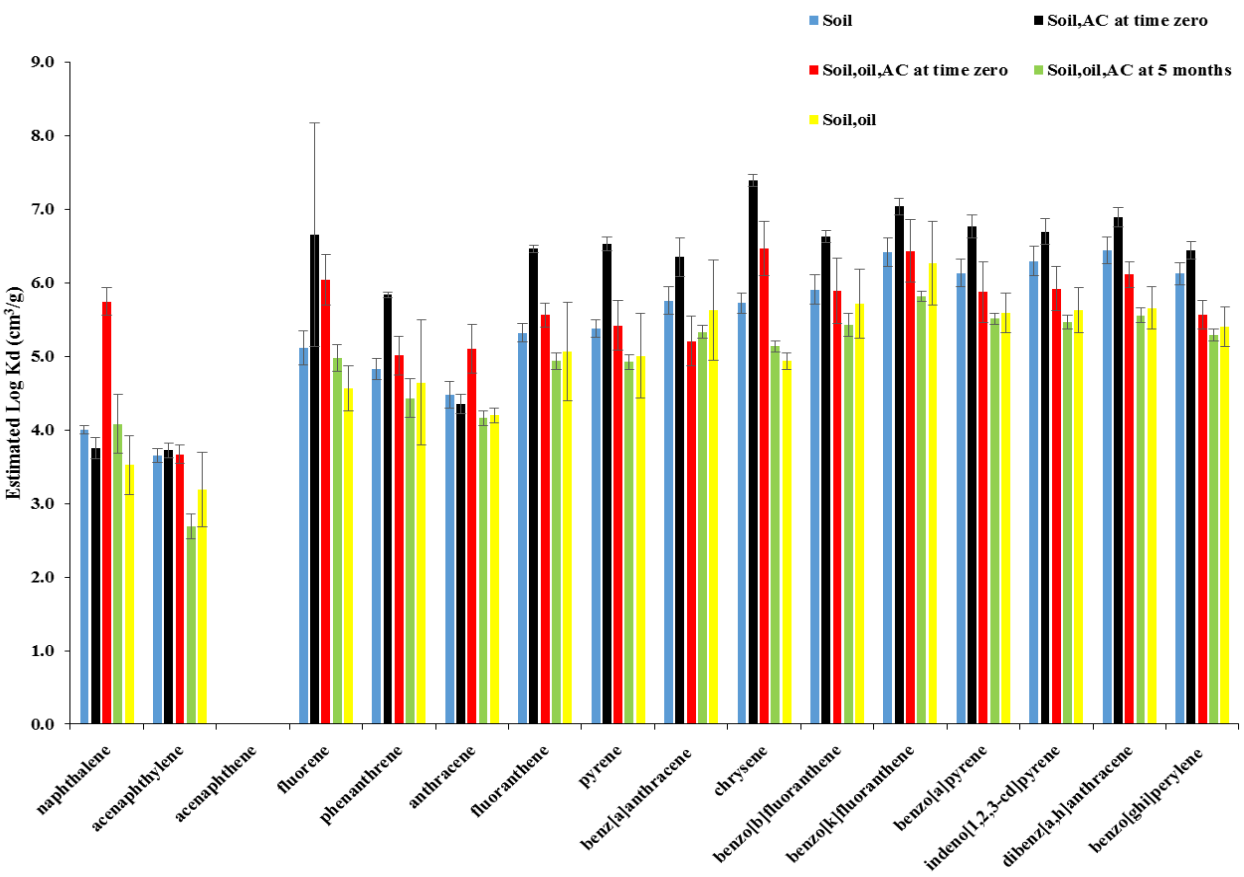

Figure S4. Estimated Log K_d values of 16 US EPA polycyclic aromatic hydrocarbons obtained using P.E and soil PAHs concentrations. Error bars: represent 1 standard deviation (SD=3) from the mean of three replicates

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